

## Expression of *trk* in MAH cells lacking the p75 low-affinity nerve growth factor receptor is sufficient to permit nerve growth factor-induced differentiation to postmitotic neurons

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**ABSTRACT** We have transfected MAH cells, an immortalized sympathoadrenal progenitor cell line, with a plasmid encoding the 140-kDa Trk protein, a nerve growth factor (NGF) receptor with protein-tyrosine kinase activity. NGF promotes neurite outgrowth and proliferation from such cells, indicating that Trk is sufficient to mediate such responses in the absence of significant levels of the endogenous 75-kDa low-affinity NGF receptor (p75). These initial NGF responses are indistinguishable from those evoked by basic fibroblast growth factor (bFGF). However, NGF is sufficient to promote terminal differentiation of a  $\approx 8\%$  of *trk*-transfected MAH cells to postmitotic, NGF-dependent neurons, whereas all cells eventually die in medium with bFGF. Other environmental signals (such as depolarization or ciliary neurotrophic factor) can cooperate with NGF to enhance production of postmitotic NGF-dependent neurons in *trk*-transfected MAH cells. The terminal differentiation of sympathetic neurons thus involves sequential and cooperative actions of different growth and neurotrophic factors, as well as cell-intrinsic changes in the response to these factors.

In vertebrates, neuronal differentiation and survival are controlled by polypeptide growth and neurotrophic factors, as well as by cytokines (1, 2). Studies of normal and immortalized progenitors in the sympathoadrenal lineage of the rat have demonstrated a relay or cascade of factors that controls neurogenesis (for review, see ref. 3). Basic fibroblast growth factor (bFGF) can act to promote the proliferation and initial neuronal differentiation of the cells but cannot support their survival (4–6). However, in chromaffin cells bFGF appears to induce a dependence upon nerve growth factor (NGF), which in turn supports the subsequent maturation and survival of postmitotic sympathetic neurons (5). Thus, bFGF can act as a “progression factor,” while NGF acts as a trophic factor. In addition, proliferating sympathetic neuroblasts respond to mitogens such as insulin-like growth factor I (7–9), which may act as interim survival factors together with other neurotrophins such as NT-3 (10, 11).

While the actions of neurotrophic factors on neural precursor cells have been studied intensively, less is known about the regulation and function of the membrane receptors that are required to respond to these factors. Two receptors for NGF have been identified: the receptor tyrosine kinase Trk and the low-affinity NGF receptor p75 (for reviews, see refs. 12 and 13). Several lines of genetic and immunologic evidence have suggested that p75 is not necessary for a functional response to NGF and that Trk is sufficient for some functional responses in nonneuronal cells (for reviews, see refs. 12–15). However, it has been more difficult to establish whether Trk is sufficient for biological responses to

NGF in neuronal cells, because most neuronal cell lines used in studies of Trk function, such as PC12 rat pheochromocytoma cells and their mutants, contain endogenous p75 (16, 17). MAH, a *v-myc*-immortalized sympathoadrenal progenitor cell line that does not respond detectably to NGF (6), expresses undetectable levels of p75 protein as assayed by immunocytochemistry (18) or affinity crosslinking (19). With reverse transcription (RT)-PCR, an extremely low level of p75 mRNA is detected, corresponding to 1/1000th the level in PC12 cells (19). MAH cells grown in the absence of dexamethasone also express little or no *trk* mRNA (18, 19).

Previously, we demonstrated that membrane depolarization of MAH cells with 40 mM KCl induced expression of endogenous *trk* mRNA, but not p75 protein (18). Depolarized MAH cells also exhibited a functional response to NGF (18). However, it remained unclear whether the induction of *trk* expression was sufficient to account for the effects of NGF on these cells. For example, depolarization might also induce other components of the signal transduction machinery which are necessary for a functional response to NGF. To examine the function of Trk in MAH cells lacking significant levels of p75 without the complicating influence of depolarization, we have introduced a human *trk* cDNA into these cells by transfection and studied their responses to NGF.

### MATERIALS AND METHODS

**Culture and Transfection of MAH Cells.** MAH cells were maintained in L15-CO<sub>2</sub> medium with 10% fetal bovine serum and 5  $\mu$ M dexamethasone (6). In all experiments involving differentiation of the cells, dexamethasone was removed from the medium. MAH cells were transfected by the calcium phosphate precipitation procedure with an expression plasmid containing a human Trk cDNA (20) and the histidinol dehydrogenase gene as a selectable marker (21). This human cDNA encodes the A<sub>I</sub> isoform of Trk, which is expressed predominantly in nonneuronal tissues (34); the recently described TrkA<sub>II</sub> isoform is neuron-specific but both isoforms have identical activities in fibroblast survival assays (34). Because of the low transfection efficiencies obtained ( $\approx 0.1\%$ ), it was not practical to perform transient-expression assays or to isolate individual clones of stably transfected cells. Rather, pools of histidine-resistant cells were expanded until sufficient numbers were achieved to perform an experiment (2–3 weeks), and the cell population was then used in its entirety. Each experiment was performed at least twice with independently generated pools of transfectants. NGF was routinely used at 50 ng/ml, and ciliary neurotrophic factor (CNTF) at 10 ng/ml.

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Abbreviations: bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; LDH, lactate dehydrogenase; MAH[*trk*<sup>hu</sup>] cells, MAH cells transfected with human *trk* A<sub>I</sub> cDNA; NGF, nerve growth factor; RT, reverse transcription.

**PCR Assays.** *trk* mRNA in transfected MAH cells was assayed by a semiquantitative RT-PCR method (22). The oligodeoxynucleotide primers used were primer 1 (5'-ATGAGACCAGCTTCATC-3'), primer 2 (5'-CTCCTTCTC-GCCAGTGG-3'), and primer 3 (5'-GCTCCCACTTGAGAA-TG-3'). Primer 2 detects an alternatively spliced exon (34) present in the form of rat *trk* mRNA expressed in PC12 and depolarized MAH cells, but not in the human *trk* cDNA used for transfection (see Fig. 1A *Inset*). PCR conditions were as follows: denaturation, 1 min at 96°C; annealing, 1 min at 52°C; extension, 1 min 10 sec at 72°C. Amplification was performed for 35 cycles, which was within the linear range of the assay.

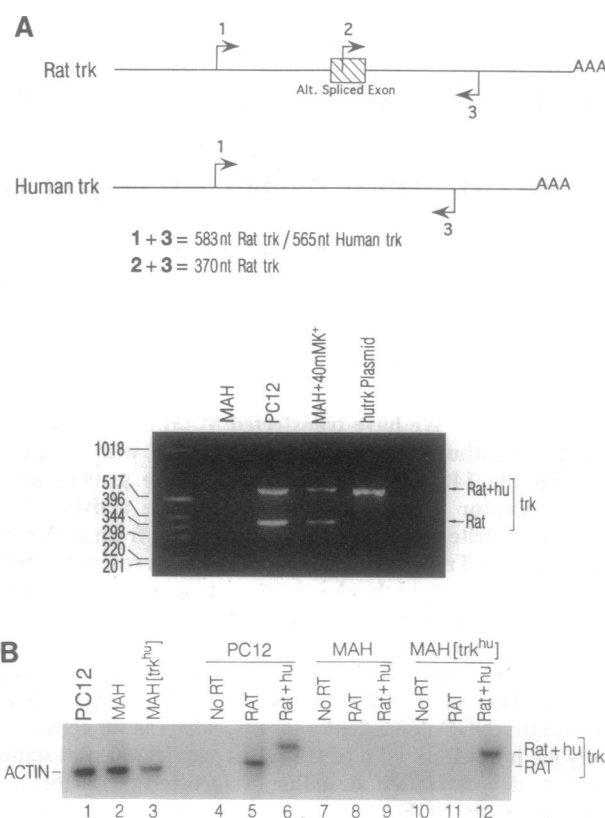
**Biological Assays.** Cell number was determined with a lactate dehydrogenase (LDH) colorimetric assay (23). Alternatively, the number of cells or cell clusters was directly determined by counting microscopic fields. Neurite outgrowth was scored with a minimum length of 1 cell body diameter as a cutoff. [<sup>3</sup>H]Thymidine labeling was performed by incubating cultures for 4 hr with [methyl-<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml; 1  $\mu$ Ci = 37 kBq) and then assaying trichloroacetic acid-precipitable radioactivity. Immunocytochemical labeling was performed as described elsewhere (6).

## RESULTS

**MAH Cells Expressing Exogenous *trk* mRNA Display Functional Responses to NGF.** Expression of endogenous and transfected *trk* mRNA in MAH cells was measured by RT-PCR (Fig. 1). We used one set of primers that detected both rat and human *trk* mRNAs (Fig. 1A, primers 1 and 3), and another set (primers 2 and 3) that detected an alternatively spliced neural-specific isoform of rat *trk* mRNA [*trk*<sub>AII</sub> (34)] expressed in PC12 cells (Fig. 1A *Inset*, lane PC12; Fig. 1B, lane 5) and depolarized MAH cells (Fig. 1A *Inset*, lane MAH + 40 mM K<sup>+</sup>). Primers 2 and 3 do not detect human *trk*<sub>A1</sub> message encoded by the expression plasmid (Fig. 1A *Inset*, lane hutrk Plasmid). Neither endogenous rat nor human *trk*<sub>A1</sub> sequences were detected in control MAH cells grown for 24 hr in the absence of dexamethasone (18) (Fig. 1B, lanes 8 and 9). However, in MAH[*trk*<sup>hu</sup>] cells a 565-bp PCR product was detected as expected (Fig. 1B, lane 12), and no endogenous rat *trk* mRNA was detected (Fig. 1B, lane 11).

Pools of stably transfected MAH[*trk*<sup>hu</sup>] cells displayed two short-term responses to NGF (visible in 24–36 hr): neurite outgrowth and extensive proliferation (Figs. 2A and 3A and data not shown). Such responses were dependent upon addition of NGF and were not observed in cells transfected with human p75 or vector alone (Fig. 2D; Fig. 3A and B; data not shown). Moreover, NGF appeared to have a trophic effect, as few cells remained after 5 days without NGF treatment (Fig. 3B and D). Importantly, the short-term NGF responses observed in MAH[*trk*<sup>hu</sup>] cells were similar to those seen in depolarized MAH cells (Fig. 2B). This suggests that the induction of endogenous *trk* is sufficient to account for the NGF responsiveness displayed by depolarized MAH cells. As in depolarized MAH cells (18), no expression of endogenous p75 was detected by immunolabeling in MAH[*trk*<sup>hu</sup>] cells even after 5 days (data not shown), confirming that *trk* expression was sufficient for a biological response to NGF in the absence of p75 in these neuronal cells. Finally, over the first 3 days, the responses to NGF in MAH[*trk*<sup>hu</sup>] cells were similar to those observed in response to bFGF (Fig. 2C and Fig. 3C and D). This suggests that when expression of *trk* is forced in an immature neuronal precursor cell, NGF can act on such cells as a mitogen (24) and differentiation factor, like bFGF.

To examine in more detail the relative contributions of *trk* expression to proliferation and survival, we performed [<sup>3</sup>H]thymidine labeling experiments. Like bFGF, NGF stimulated thymidine incorporation into MAH[*trk*<sup>hu</sup>] cells over



**FIG. 1.** Expression of human *trk* mRNA in transfected MAH cells. (A) Location of the two primer sets used for *trk* RT-PCR. Primer 2 anneals to an alternatively spliced minixion (34) expressed in rat PC12 cells and depolarized MAH cells (*Inset*); note that the two bands detected in these cells do not represent two different transcripts but, rather, represent two different-size PCR products derived from the same cDNA template. The minixion is absent from the human *trk* cDNA used (hutrk plasmid). Primers 1 and 3 hybridize equally well to both human and rat *trk* sequences (J.M.V., unpublished data; see B, lane 6). (B) RT-PCR with  $\beta$ -actin primers (lanes 1–3), *trk* primers 2 and 3 (lanes 4, 5, 7, 8, 10, and 11), or *trk* primers 1 and 3 (lanes 6, 9, and 12). Approximately 1/20th the amount of cDNA was used for the actin amplification as for the *trk* amplification; the number of cycles was kept constant at 35. Note that in human *trk*-transfected MAH cells (MAH[*trk*<sup>hu</sup>] cells) a 565-bp human *trk* RT-PCR product (lane 12) but no endogenous rat product (lane 11) was seen; neither set of primers yielded a product in nontransfected MAH cells (lanes 8 and 9; see also *Inset* in A). Low levels of endogenous *trk* mRNA can be detected in control MAH cells grown in medium with dexamethasone (18); however, these levels are insufficient for a functional response to NGF. “No RT” indicates controls in which reverse transcriptase was omitted from the reaction.

the first 3 days of culture (Fig. 3C). After that time, thymidine incorporation appeared to decline. Examination of total cell number in parallel cultures by LDH assay revealed that much of this decline was due to cell loss (Fig. 3D), although in the presence of NGF, it also reflects the accumulation of post-mitotic cells (Fig. 3C and D). That some of the cell loss reflected apoptosis is supported by the fact that it was inhibited by aurintricarboxylic acid (data not shown), a nuclease inhibitor that blocks programmed cell death in sympathetic neurons and PC12 cells (25). However, the rate of cell loss in bFGF-containing medium was greater than that in NGF-containing medium for the *trk*-transfected cells (Fig. 3D) and cell number in medium with NGF eventually stabilized, while in medium with bFGF it decreased to zero (see below), reflecting the fact that NGF acts as a survival factor for at least some of the *trk*-expressing cells, while bFGF does not.

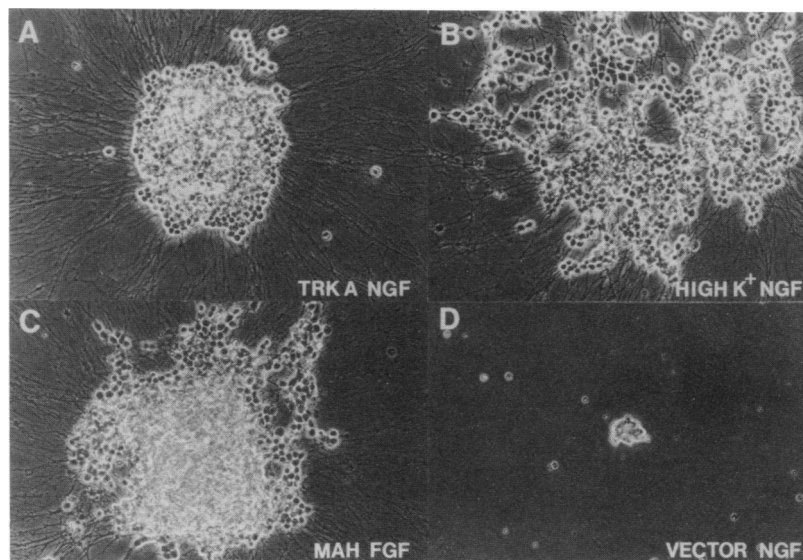


FIG. 2. NGF promotes proliferation and neurite outgrowth from MAH[*trk<sup>hu</sup>*] cells. Representative fields are shown from cultures treated for 5 days under the indicated conditions. Quantification is presented in Fig. 3. (A) MAH[*trk<sup>hu</sup>*] cells exposed to NGF. (B) Nontransfected MAH cells depolarized with 40 mM KCl [which induces endogenous *trk* (18)] and exposed to NGF. (C) Nontransfected MAH cells exposed to bFGF (10 ng/ml). (D) MAH cells transfected with expression vector lacking the *trk* cDNA insert and exposed to NGF. These same cultures were fixed and immunostained for p75; none showed any detectable p75 (data not shown).

**Expression of *trk* Is Sufficient for Terminal Differentiation in Some Cells.** Previous work showed that treatment of MAH cells with bFGF induced neurite outgrowth in virtually all cells but was insufficient to support their terminal differentiation (6). In the presence of both bFGF and NGF, however, a very small proportion of MAH cells (0.01–0.2%) were able to differentiate to NGF-dependent, postmitotic neurons (6). These observations raised the question of whether NGF alone was sufficient to promote terminal differentiation in *trk*-expressing MAH cells, or whether bFGF was still necessary. In addition, we wondered whether the forced expression of high levels of *trk* mRNA would increase the efficiency of terminal differentiation beyond that seen in medium with bFGF and NGF. To address these issues, we examined MAH[*trk<sup>hu</sup>*] cells after long-term (14-day) exposure to NGF.

Following the period of cell death that occurred after the first week of exposure to NGF (Fig. 3D), the number of MAH[*trk<sup>hu</sup>*] cells eventually stabilized. By 14 days, these cells had undergone further maturation, exhibiting the cell soma hypertrophy, enlarged clear nucleus, and prominent nucleolus characteristic of normal sympathetic neurons (Fig. 4A). These MAH-derived neurons did not divide further, suggesting that they had become postmitotic. The neurons expressed tyrosine hydroxylase (Fig. 4B), like undifferentiated MAH cells, indicating that they had maintained their sympathoadrenal character. In addition, the cells now expressed endogenous p75 (data not shown), which first became detectable (by immunocytochemistry) at days 10–11. Most important, the survival of these cells was now dependent upon NGF; upon withdrawal of the neurotrophic factor the cells died (data not shown). Taken together, these data indicate that NGF is sufficient to induce not only initial neuronal differentiation but also maturation of MAH cells to NGF-dependent postmitotic neurons, when expression of *trk* is forced in these cells.

Forced expression of *trk* significantly improved the efficiency of generating postmitotic neurons from MAH cells: whereas only 0.25% of normal MAH cells exposed to bFGF plus NGF survived after day 5 to become postmitotic neurons,  $\approx 8\%$  of MAH[*trk<sup>hu</sup>*] cells exposed to NGF alone made this transition (Table 1), a 32-fold enhancement. This suggests that an insufficient induction of endogenous *trk* is in part

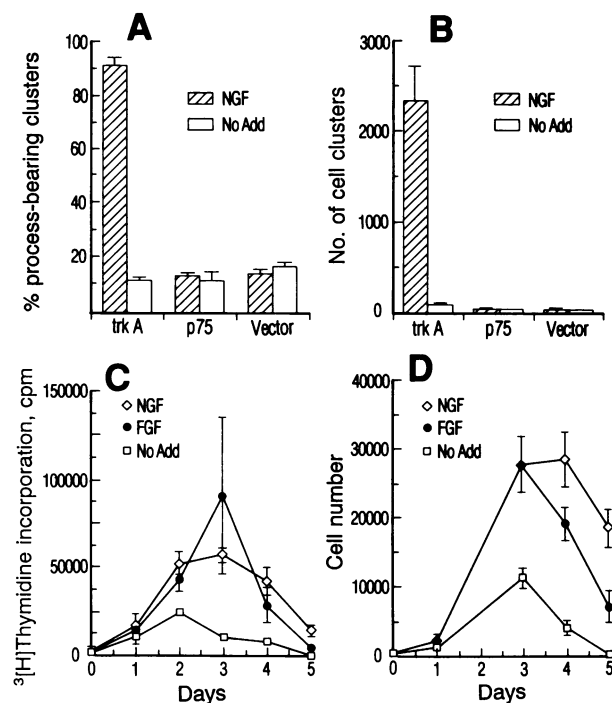


FIG. 3. Quantification of NGF responses of MAH[*trk<sup>hu</sup>*] cells. (A and B) The percentage of process-bearing cell clusters (A) or the total number of cell clusters (B) was determined after 5 days by direct microscopic counting. Note that no NGF response is observed in MAH cells transfected with p75 cDNA but not *trk* cDNA. Data represent the mean  $\pm$  standard deviation of triplicate determinations from one of several independent experiments. (C) [ $^3\text{H}$ ]Thymidine incorporation was performed by incubating cells with [ $^3\text{H}$ ]thymidine for the last 4 hr of each 24-hr period indicated; sister plates were used for each time point. Total thymidine incorporation was determined by scintillation counting. (D) Cell number was determined by LDH colorimetric assay (23). Note that at days 4–5, [ $^3\text{H}$ ]thymidine incorporation in bFGF-treated cells is lower than in NGF-treated cells (C), and total cell number is higher in NGF-treated cells (D), reflecting the survival-promoting activity of NGF. All cells eventually die in medium with bFGF, whereas in medium with NGF the population stabilizes (see Fig. 4 and Table 1).

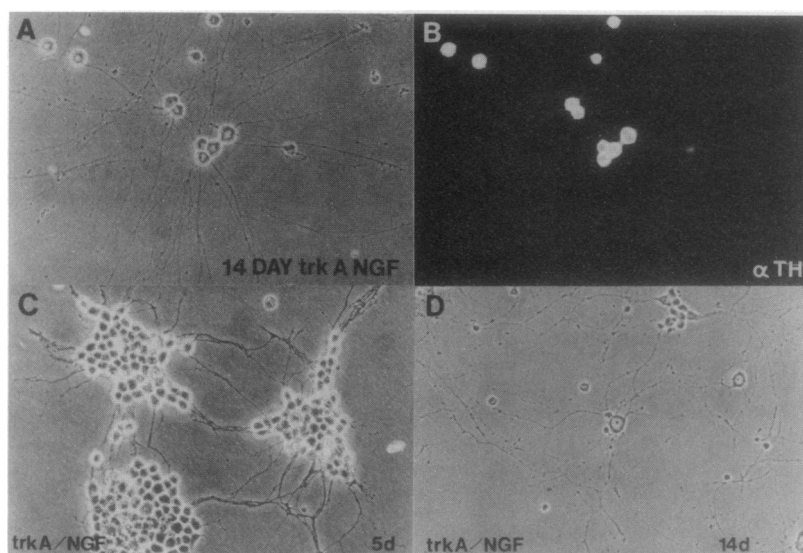


FIG. 4. *trkA* expression is sufficient, but not limiting, for terminal differentiation of MAH cells in response to NGF. (A and B) MAH[*trk*<sup>hu</sup>] cells exposed to NGF for 14 days; phase-contrast (A) and anti-tyrosine hydroxylase ( $\alpha$ TH) immunofluorescence (B) are shown for the same microscopic field. (C and D) Sequential photographs of the same culture of MAH[*trk*<sup>hu</sup>] cells exposed to NGF for 5 days (C) and 14 days (D). Note that although some terminally differentiated neurons survive to 14 days, they still represent a small proportion of the cells present at day 5 ( $\approx$ 8%; see Table 1).

responsible for the low efficiency of terminal differentiation of normal MAH cells exposed to bFGF plus NGF. However, the fact that the recovery of postmitotic neurons from MAH[*trk*<sup>hu</sup>] cells exposed to NGF alone was still only 8% (Fig. 4 C and D; Table 1) suggests that the amount of *trk* expression is not the only factor limiting successful terminal differentiation of these cells.

**Additional Factors Can Collaborate with NGF to Enhance Terminal Differentiation of MAH Cells.** Both membrane depolarization (18) and CNTF (N.I., T. Boulton, Y. Li, J.M.V., S. Birren, D.J.A., and G.D.Y., unpublished work) have been shown to be capable of collaborating with bFGF and NGF to enhance the terminal differentiation of normal MAH cells to postmitotic neurons. Depolarization has also been shown to interact synergistically with growth or neurotrophic factors in other neuronal systems (26, 27). Although CNTF and depolarization appear to act through different pathways (N.I., T. Boulton, Y. Li, J.M.V., S. Birren, D.J.A., and G.D.Y., unpublished work), the data raise the questions of whether these factors interact specifically with bFGF and whether their effect is simply to enhance the induction of endogenous *trk*. To address these issues, we examined the effect of these factors on terminal differentiation of MAH[*trk*<sup>hu</sup>] cells exposed to NGF in the absence of bFGF.

Table 1. Environmental factors cooperate with NGF to boost the efficiency of converting MAH [*trk*<sup>hu</sup>] cells to postmitotic neurons

Condition	% efficiency of neuron generation*
NGF	8.5 $\pm$ 0.35
NGF + CNTF	25 $\pm$ 4.3
NGF + 40 mM KCl	43 $\pm$ 5.2
NGF + CNTF + 40 mM KCl	70 $\pm$ 6.0

NGF was used at 50 ng/ml, and CNTF at 10 ng/ml.

\* Calculated by counting the total number of neurons on the plate at day 14 (when the number of cells had stabilized) and dividing that by the number of cells present at day 5 (the time at which the maximum number of cells was present in the culture following the initial proliferative response to NGF). This fraction is expressed as a percentage. This parameter underestimates the recovery of neurons relative to the day of plating, because of the substantial proliferation that occurs in the first 5 days (see Fig. 3D). Numbers represent the mean  $\pm$  range of duplicate determinations.

Both CNTF and depolarization substantially increased the percentage of NGF-treated MAH[*trk*<sup>hu</sup>] cells present at day 5 that survived to day 14 as postmitotic neurons (Table 1). The effect of combining these two treatments appeared to be additive, yielding a 70% efficiency of terminal differentiation. (However, since both CNTF and depolarization are antimitotic for MAH cells, some of this increased percent recovery reflects a depressed cell number at day 5.) These data indicate that the synergistic effects of CNTF and depolarization are not specific to bFGF but can be observed with NGF if the cells are forced to express *trk*. The total levels of *trk* mRNA (human plus endogenous rat *trk*) expressed at day 5 were comparable under these various conditions (Fig. 5). Although depolarization (but not CNTF) induces endogenous rat *trk* expression in MAH cells (18), the level of *trk* mRNA in depolarized cells is at least 5-fold lower than the level of human *trk* mRNA expressed in transfected cells (19). Therefore, most of the *trk* mRNA detected in MAH[*trk*<sup>hu</sup>] cells under the various conditions of Fig. 5 was probably human in origin. Since *trk* mRNA levels appear to be constant in MAH[*trk*<sup>hu</sup>] cells, an up-regulation of endogenous *trk* expression is insufficient to explain the effects of depolarization and CNTF on neuronal differentiation.

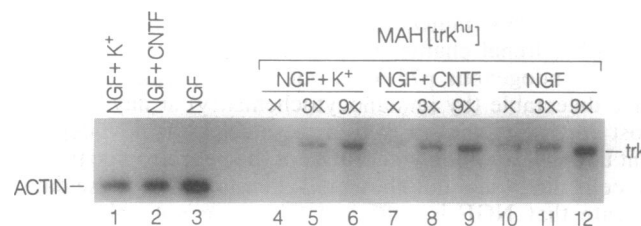


FIG. 5. Depolarization and CNTF do not increase the total amount of *trk* mRNA in MAH[*trk*<sup>hu</sup>] cells exposed to NGF. RT-PCR was performed on total RNA samples extracted from cells after 5 days of treatment in the conditions indicated above the lanes. Amplification was performed with primers 1 and 3 (Fig. 1A), which detect both human and endogenous rat *trk* sequences. X, 3X, and 9X indicate serial three-fold dilutions of the cDNA template. When normalized to the actin control (lanes 1–3), the amounts of *trk* mRNA do not differ substantially between the various conditions. K<sup>+</sup> indicates depolarized MAH cells.

## DISCUSSION

We have used MAH cells to examine biological responses to NGF in neuronal cells expressing exogenously introduced *trk* but not p75. Two main conclusions can be drawn from this analysis. (i) The results provide further evidence that Trk is sufficient to mediate biological responses to NGF in neuronal precursor cells lacking p75. While several lines of evidence have suggested that p75 is not necessary for neuronal differentiation or survival (28–30), these studies did not establish which NGF receptor(s) were functionally involved. Our findings are consistent with recent results showing that PC12 mutants expressing a truncated form of p75 and reduced levels of endogenous p75 can still respond to NGF (31) and that human neuroblastoma cells lacking endogenous p75 can respond to NGF if transfected with *trk* (32).

(ii) The results indicate that NGF can act as a progression factor (like bFGF) as well as a maturation and survival factor for MAH cells expressing *trk*. Thus, although bFGF and NGF normally play sequential and distinct roles in the differentiation of MAH cells (6), some of these differences must reflect a change in the cellular context in which their respective receptors are expressed, rather than an intrinsic difference in the nature of the signals transmitted by the FGF and NGF receptors. These results also establish MAH cells as one of the few cell lines which exhibit progressive changes in the quality of the response to the same growth factor during differentiation. Finally, the results suggest that the low efficiency of terminal differentiation of MAH cells to post-mitotic, NGF-dependent neurons in the presence of bFGF plus NGF (6, 18) is not simply due to an inefficient induction of endogenous *trk* expression but more likely due to a requirement for other environmental signals that must cooperate with NGF in this process.

Several new questions are raised by the present results. Although our data suggest that *trk* expression is sufficient for an NGF response in neuronal cells lacking p75, they do not exclude the possibility that p75 modulates this response in some manner. Recent results indicate that coexpression of p75 in *trk*-expressing MAH cells both enhances tyrosine autophosphorylation of Trk and accelerates mitotic arrest and neuronal maturation (19). The demonstration that NGF and bFGF each elicit similar early responses from MAH cells [as they do from PC12 and chromaffin cells (4, 5, 33)] begs the question of why NGF and not bFGF is able to act as a survival factor for these cells. A simple explanation is that FGF receptor expression is rapidly extinguished during neuronal differentiation; alternatively, Trk protein may activate cell survival pathways not activated by the FGF receptor. In the latter case, MAH cells would provide a useful system to examine the activity of FGF–NGF receptor chimeras on such survival responses. Finally, it should be emphasized that although MAH cells provide a convenient way to dissect signals and regulatory pathways controlling neuronal differentiation, it remains to be determined how expression of *trk* is controlled during normal sympathetic development and whether signals such as bFGF and CNTF actually function *in vivo* in the manner suggested by these *in vitro* studies.

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